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Prepared by: Dr Wendy Muir and Associate Professor Peter Williamson
University of Sydney

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Meat & Livestock Australia Limited
Locked Bag 991
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Strategies to identify and develop bioactive peptides in meat and bone meal.

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Abstract

Meat and bone meal (MBM) consists of up to 50% protein, which may contain bioactive agents capable of increasing animal growth performance. Bioactive agents identified in MBM could be refined for optimal feeding strategies. In this exploratory study to search for bioactive molecules in MBM, proteins of set molecular weight sizes were extracted from MBM sourced from low and high temperature rendering processors. These were then administered to broiler chicks to evaluate their effect on chick growth. When delivered to the chicks *in-ovo*, no growth or weight benefit was observed. However, when fed to chicks in the first week post-hatch, proteins/peptides of <100kDa from MBM rendered at the lower temperature promoted increased chick-body and breast-weights. Pretreatment of the protein group with a protease did not generate any additional benefits. In conclusion, the findings support proof of the principle that MBM contains growth-positive protein-derived bioactive agents. Additional investigations are now required to further fractionate this bioactivity to identify the bioactive proteins/peptides present and to determine whether they can be enriched and prepared as a feed supplement to benefit chick-growth.

Executive Summary

MBM, the rendered by-products of the meat industry, is a good source of protein, calcium and phosphorus and is routinely used as a fertilizer or included in diets formulated for pets and livestock. However the inclusion of MBM in livestock diets has declined in recent times due to the presence of BSE in ruminants in Europe. This has restricted the inclusion of MBM to the diets of non-ruminant animals. Further, some challenges have been encountered while formulating diets containing MBM for non-ruminants due to the between batch variability in its quality, particularly in terms of its crude protein levels, amino acid, calcium, phosphorus and ash content. Therefore alternative uses for MBM are being explored.

In recent times there has been an accumulation of knowledge about bioactive peptides, that is proteins which facilitate more than the basic nutritional requirements of an animal, as they have the ability to enhance growth and bolster physiological function. Bioactive agents have been identified in high protein products such as milk and eggs. Therefore with approximately 50% protein content in MBM it may also be considered a candidate source of bioactive substances.

In determining whether MBM contains bioactive proteins or peptides the following questions were posed:

1. Which proteins/peptides are present in MBM
2. Does the rendering process affect the presence and viability of these proteins and peptides.
3. Do these peptides possess bioactivity, with the capacity to improve the health, nutrition, and growth of broiler chicks.
4. Can dietary supplements (such as proteases) enhance the bioavailability and or bioactivity of these peptides

Throughout the research project two sources of MBM, one from a high temperature rendering facility (identified as Throsby) and the second from a lower temperature rendering facility (identified as Rockdale), were assessed. This approach was chosen to represent the type of variation in peptides that could be expected from different rendering facilities in Australia. In interpreting the results from these two facilities we were also mindful of likely differences in the source material in each plant – with the former generally processing older cattle with higher bone content, compared with the latter.

Proteins and peptides were identified in both sources of MBM, with collagen type -1 being the predominant protein in both samples. However MBM derived from the higher temperature Throsby facility had fewer resolved protein/peptide types, than in the lower temperature Rockdale MBM. This may in part be a reflection of the raw materials used at each facility as outlined above.

Proteins and peptides from both of the MBM sources were extracted into fractions of pre-determined ranges based on molecular weight, for example with the Throsby samples <50kDa or >50kDa and with Rockdale <3kDa, 3-100kDa and >100kDa. These were then

administered orally to chicks, either *in-ovo*, that is 3 days prior to hatch, or during the first week post-hatch, with subsequent assessment of chick growth in terms of bird weight, breast muscle (the prime cut of chicken meat) weight and intestinal weight and length.

None of the tested peptide fractions from either of the samples of MBM impacted chick growth following their *in-ovo* administration. However when administered to chicks during their first week post-hatch, the Rockdale 3-100kDa peptide fraction did positively influence chick performance. While not being statistically significant, in one study these chicks had body weights notably higher than the control chicks at 2, 3 and 4 weeks of age. Similarly the breast weight of these birds was notably increased. In a second study Rockdale 3-100kDa treated birds had significantly longer jejuna and ileum at 4 weeks of age. In these same birds mean body weight was notably higher at 3 weeks and breast weight was increased at 4 weeks of age compared to the control birds.

The extracted fractions were prepared fresh for each experiment and subsequently there was some quantitative variability in protein dosage used between chick experiments. This is likely to have resulted in some of the variation in the *in-vivo* outcomes between chick experiments. Therefore to overcome any dose-dependent effects further *in-vivo* exploration using a single extraction preparation is required. Studies with close regulation of protein/peptide dosage would have two benefits. They would enable optimization of the feed regimes for maximal growth and secondly they would give a definitive dose-dependent proof of this bioactive response.

A final evaluation included the pretreatment of the peptide fractions with protease cocktail which may release other bioactive peptides from the initial peptide fractions. No trends were observed in the performance of birds through to 4 weeks of age following administration of these protease treated peptide fractions during the first week after hatch.

These studies have demonstrated proof of principle that some proteins and/or peptides in MBM possess growth enhancing bioactivity. In this project bioactive agents were extracted in a water-soluble protein and/or peptide fraction of less than 100kDa molecular weight from low temperature rendered MBM, but not from the higher temperature rendered MBM. Further studies are required to enrich, purify and identify the bioactive agents, and to test them in a dose-varied feeding strategy for maximal growth enhancement and full realization of their benefit for the meat industry. A cost-benefit analysis would be required to establish the financial practicalities of sourcing this material from rendered material. Another possible supply of bioactive agents is the stickwater of low temperature rendering facilities. Containing between 2-4% proteins its analysis is warranted. However such testing was beyond the scope of the current project.

In these studies the delivery of the Rockdale 3-100kDa fraction to chicks immediately after hatch demonstrated that some of the proteins/peptides in this fraction possess bioactivity that can improve the growth performance of meat chickens. As discussed above closer characterization and testing of this fraction, including between MBM batch comparison and dose titration analysis, is recommended. Identification of the bioactive proteins and peptides within this fraction would enable their targeted extraction and concentration specifically for administration to broiler chickens. The rendering industry then has the opportunity to retrieve and enrich bioactive agents from appropriate sources of MBM for use as a high quality bioactive material. When administered to meat chickens, increased growth rates and

breast muscle deposition would benefit the poultry industry, while also expanding the utilization of MBM with positive outcomes for the rendering industry.

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Background

Meat and bone meal (MBM) is a by-product of meat production that is used as a feed additive in the diet of livestock. By definition, MBM consists of the rendered products from mammalian offal. “Hard offal includes bone, heads, hooves, skin cartilage and connective tissue” and “soft offal includes some skeletal muscle, lungs and the gastrointestinal tract” (Anon 1997a). Typically hair, wool and hides are excluded, except if they are attached to the hoof or head (Aird and Spragg 2003).

The principle of rendering is to treat raw materials that are not suitable for human consumption to produce meat and bone meal by-products that can be used either as fertiliser, stock or pet feeds. In this context MBM is an important source of protein, and the minerals calcium and phosphorus (Hamilton, 2002). The rendering process includes fragmenting and homogenising the material prior to it being heated. Heating is designed to remove the water and to kill the majority of microbial agents. The fat is then removed from the solid material, and is processed to produce tallow or grease (Garcia 2007).

Due to the presence of bovine spongiform encephalopathy (BSE) in ruminants in Europe the use of MBM in the diet of livestock has more recently been limited to its inclusion in feeds for non-ruminant animals, including poultry. However, there is some between batch variation in MBM quality, especially in terms of its levels of crude protein, amino acid, calcium, phosphorus and ash (Parsons *et al* 1997; Ravindran *et al* 2002; Bryden *et al* 2009) that has caused concern for poultry nutritionists when formulating diets that include MBM. To some degree this has reduced the usage of MBM in poultry diets. Further, depending on the markets, the rate of inclusion of MBM in poultry diets has been influenced by the price of MBM relative to vegetable protein sources, particularly imported soybean meal (Personal communication, Associate Professor Peter Selle, University of Sydney). This, together with the increased regulatory restrictions placed on the use of MBM in animal diets, has initiated investigation into other opportunities for the use of MBM. Recent work indicating the presence of bioactive substances in cooked beef meat and collagen (the main protein in MBM) has increased interest in the proposal that the dietary protein component of MBM may contain bioactive substances that could have application for enhancing animal production, especially chicken meat production.

Dietary proteins are one of the essential nutrients required by the body for maintenance and growth. However some of these proteins may have additional benefits through demonstrated bioactivity. This may be a property of the protein itself, or of smaller peptide sequences found within the parent protein. Meisel (2007) defined bioactive compounds as “substances, both nutrient and non-nutrient, which may exert regulative activities in an organism beyond basic nutrition”. Bioactive agents have a wide range of nutritional, functional and physiological properties. Typically they are specific protein fragments, normally compromising of 2-20 amino acid residues (Srinivas and Karim 2009). Bioactive substances may be encoded within a larger protein molecule, requiring a trigger to initiate their release and activation. Once released, the bioactive can influence various aspects of body function, including overall health (Kitts and Weiler 2003)

Kitts and Weiler (2003) emphasise the difference between proteins with bioactivity that are found naturally in foods for example, milk immunoglobulins and the bioactive substances that are released from the parent or precursor protein by digestion, for example casein-

derived phosphorylated peptides. Bioactivity may also be derived from other compounds in the diet, such as amino-acid oligomers, protein fragments and growth factors.

Bioactive agents have been identified in a variety of foods of both animal and plant origin. Milk proteins and their constituent peptides are the most extensively studied food-derived proteins and bioactive peptides respectively, and the literature in this area has been regularly reviewed (Shah 2000; Pellegrini 2003; Clare *et al* 2003; Smithers 2008; Haque *et al* 2009). Bovine milk and avian eggs contain an array of bioactive peptides present in their active form, and many latent peptides that are inactive within the protein sequence and require enzymatic proteolysis for release. Bioactive proteins have also been identified in other animal proteins, such as collagen (Li *et al* 2007) hen eggs (Sakanaka and Tachibana 2006; Mine 2007; You *et al* 2010) and fish (Fujita and Yoshikawa 1999; Medenieks and Vasijevic 2008). Bioactive peptides have been identified in many food sources of both vegetable and animal origin and can have a wide range of nutritional, functional and active properties. Of particular interest is the variety of bioactive peptides that have been identified with nutraceutical and medicinal properties of benefit and application for human and veterinary health and medicine.

Garcia (2007) discussed the possibility of using MBM as a source of bioactive components with nutraceutical characteristics. To this end Garcia and Phillips (2008) examined the protein composition of MBM sourced from rendering facilities from across the USA and Canada to better understand its characteristics. They found soft tissue protein to be predominant and of that collagen was most common. In mammals up to 30% of total body protein is collagen, which includes the bulk of bone, skin, tendon, loose connective tissue and muscle protein. Using the Bioactive Peptides (BIOPEP) database to divide food proteins into families of similar bioactivity Dziuba and Darewicz (2007) identified the collagen family as containing peptides with chemotactic and anti-thrombotic activity, as well as having a regulatory role over the function of the gastric mucosa. Additionally, Li *et al* (2007) identified anti-oxidative peptides in collagen derived from pig skin.

In the absence of any direct reports of bioactive substances in MBM investigation of the evidence of bioactive substances in meat and especially cooked meat is relevant. Details of meat-derived bioactive peptides are sparse, but antihypertensive bioactive peptides have been observed in the muscle of beef (Bauchart *et al* 2007a), chicken (Fujita *et al* 2000) and pork (Arihara *et al* 2001). Hydrolysation of meat proteins may occur with aging (and storage), processing, cooking and digestion (Arihara 2006). Bauchart *et al* (2006, 2007a, 2007b) assessed the presence of peptides in cooked beef. Bauchart *et al* (2006) identified a predominance of peptides of low molecular weight (less than 5000 Da) in beef meat when vacuum cooked (75 ^{0}C for 1.5 hours) after 2 weeks in cold storage (4^{0}C). When cooked beef meat was digested by pigs, short peptides (typically less than 2000 Da) were identified. Their amino acid sequence was found to correspond most closely with peptides known to reduce hypertension (Bauchart *et al* 2007a). Bauchart *et al* (2007b) focused on levels of the antioxidant carnosine in cooked and digested beef, and found that it could be absorbed across the intestinal wall in a minipig model. Ohba *et al* (2003a) assessed the biofunction of collagen and keratin in meat meal following its enzymatic hydrolysis, finding high levels of anti- acetyl choline esterase (ACE) activity. They also reported a high level of antioxidative activity in keratin from bovine hoof and horn waste. This was seen to reduce oxidative damage in rats when included in their diet (Ohba *et al* 2003b).

The variability seen in the nutritional quality of MBM may also generate variations in the availability and quality of bioactive substances derived from MBM. Variation in the nutritional value is due to two main factors. Differences in the starting raw source materials, including the species, relative contributions from hard and soft offal, as well as the age and condition of the animals involved (Skurray and Herbert 1974; Johnson and Parsons 1997; Parsons *et al* 1997; Ravindran *et al* 2002) and the rendering conditions used for processing (Batterham *et al* 1986; Wang and Parsons 1998), will affect the nutritional value of MBM. Therefore in the studies presented within this project, two sources of MBM, using different raw material – in terms of animal age, and different temperature conditions during the rendering process, were utilised.

To assess whether the delivery of bioactive substances derived from MBM may deliver nutraceutical and developmental benefits to meat (broiler) chicks, an understanding of the processes of early chick development and muscle deposition is required.

The continued improvement in feed conversion and lean muscle deposition in broiler chickens is primarily due to genetic selection and improved nutrition (Dozier *et al* 2008), and has kept chicken meat competitively priced. This has increased the demand for chicken meat and processed chicken products. Approximately 60% of the value of the chicken at processing comes from the breast meat (Wilkinson and Scott 2006) with 17 to 25% of the total body weight of the chicken being attributed to breast meat yield (Havenstein *et al* 2003). Therefore the production of birds with a high breast muscle yield supports increased profitability.

The development of muscle fibres, that is hyperplasia, occurs during chick embryogenesis and continues during the first week post hatch (Halevy *et al* 2003). After this point muscle fibre numbers do not increase, however, muscle growth can be achieved by hypertrophy, that is increasing the size of muscle fibres. Hypertrophy involves the proliferation of satellite cells, which either bind to existing muscle fibre cells or combine with each other to form new muscle fibres.

Wilkinson and Scott (2006) were interested in using *in-ovo* feeding, that is supplying exogenous nutrients to the chick embryo prior to hatch, as a means of increasing hyperplasia and breast muscle yield in broiler chickens. The results of their study showed a positive effect of *in- ovo* feeding to late term embryos (from day 16 – 18 embryonation) on body weight at hatch, but the degree of improvement varied depending on the bioactive agent being fed. The authors did not expand on the bioactive agents administered however Banja and Mandal (2005) and Banja *et al* (2008) reported increased body weights at 21 days of age after *in-ovo* feeding of amino acids and glucose respectively. Coles *et al* (1999) found varying effects of feeding peptide YY *in-ovo*, where the average body weight of the peptide YY treated birds were heavier at one week of age, compared with the control birds, but this was not evident at both 3 and 6 weeks of age.

Satellite cell proliferation is the basis of hypertrophy. Mozdziak *et al* (1997) suggested that inadequate nutrition at hatch restricted breast muscle yield by limiting satellite cell activity. This was confirmed by Halevy *et al* (2000) who demonstrated that delayed feeding post-hatch reduced satellite cell myogenesis, whereas early access to feed induced satellite cell proliferation. More generally Uni and Ferket (2004) present an overview of reports to

demonstrate that immediate access to feed at hatch, termed early feeding, reduced early mortalities, increased body weights and muscle production and improved immune function.

For the first three days post-hatch the chick will continue to draw on the yolk while it adjusts to the solid diet. The high energy needs of the chick for hatching and also during the early post-hatch period will be drawn initially from the yolk, and then through access to the diet (Uni and Ferket 2004). Additional energy needs during this time may also be sourced from muscle protein, which has a negative impact on chick growth and development. Therefore early access to the diet is critical to support the nutritive needs of the chick. Interestingly, Uni and Ferket (2004) hypothesise that *in-ovo* feeding may actually stimulate appetite and the initiation of feed intake immediately after hatch, which will also promote chick survivability.

The gastrointestinal tract of late stage embryos and hatchlings is undergoing rapid development, both physically and functionally, enabling nutrient digestion and absorption (Uni and Ferket 2004). Ferket and Uni (2002) highlight the role that the consumption of the solid diet has on promoting the gastrointestinal tract and yolk sac motility, which in turn assist with the supply of nutrients from the yolk sac and diet, promoting the development of the intestine. Any setback in intestinal development and function will reduce the efficiency with which the young chick can utilize dietary nutrients, further limiting chick growth and physiological development. Studies undertaken by Noy and Sklan (1999) have shown that early access to feed specifically enhances body weight gain, the percentage of the breast muscle yield, and intestinal development. Geyra *et al* (2001) concur with this finding as they observed that birds given access to food immediately after hatch exhibited more rapid development of the intestine early in life. Dibner (1999) also showed negative consequences of feed withdrawal in young chicks, which included the delayed development of the enzymatic, metabolic and immune systems. At processing birds that received nutrients immediately after hatch were 8-10% heavier, with breast muscle yields 7-9% higher, than those that had access to water only (Noy and Sklan 1998).

If during late embryonation or early in the post-hatch period, not only dietary requirements, but also bioactive substances capable of enhancing chick development and growth are received, the chick may realize improved muscle deposition, health and productivity throughout its life. Two practical techniques for the administration of MBM-derived substances to intensively reared broiler chicks at these stages are either *in-ovo* delivery, prior to the chick hatching or via administration in the early post-hatch period. An increase in intestinal development and in the percentage of breast muscle yield, as well as enhanced chick immunity can be achieved with *in-ovo* and early feeding (Dibner 1999; Bhanja *et al* 2008; Uni and Ferket 2004; Bhanja and Mandal 2005). Therefore, in this project candidate MBM-derived bioactive fractions were delivered at either of these times for assessment of their impact on chick growth and performance.

The *in-ovo* delivery of nutrients as a means of improving embryo and young chick energy status, development, growth and survivability, has been reported in recent years. This has included the administration of substances such as carbohydrates, amino acids, minerals, glucose and growth factors (Kocamis *et al* 1998; Coles *et al* 1999; Ferket and Uni 2002; Uni and Ferket 2004; Bhanja *et al* 2004b; Tako *et al* 2004; Bhanja and Mandal 2005; Uni *et al* 2005; Bhanja *et al* 2008; Kadam *et al* 2008). Note should be made however, that there are many between study procedural variations in the timing of administration, in terms of days of

embryonation, and, the site of inoculation of these quite different materials. As early access to feed assists with the early development of the young chick (Uni and Ferket 2004), then feeding the embryo before it hatches by *in-ovo* administration should deliver an improvement in chick hatchability, more rapid development of the digestive and immune system, improved body weights and an increase in overall nutritional status of the chick.

Kocamis *et al* (1998) and Kocamis *et al* (1999) administered chicken growth hormone and recombinant human insulin-like growth factor-1 *in-ovo*, at varied stages of embyrogenesis. They found these growth factors altered aspects of chick growth and tissue development, but the specific outcomes differed according to chick gender and the age of the chick at the time of the observations.

In-ovo inoculation with sources of energy, namely carbohydrates, β-hydroxy-β-methylbutyrate (Tako *et al* 2004; Uni *et al* 2005) and glucose (Bhanja *et al* 2008), have been evaluated in terms of chick growth and intestinal development. The former delivered the nutrients into the amnion at 17.5 days embryonation, the latter delivered it into the yolk sac at 18 days embryonation. Uni *et al* (2005) identified an increase in hatch weights and breast muscle weights, which corresponded with increased intestinal development and digestive function (Tako *et al* 2004). In a similar manner Bhanja *et al* (2008) found *in-ovo* feeding with glucose increased chick and organ weights. Overall, these studies have demonstrated the potential for *in-ovo* feeding with energy dense nutrients to enhance the nutritional status of the hatchlings. This also compensates in part for the energy deprivation that the chicks may experience during hatching and the early post-hatch period.

In-ovo administration of various amino acids or amino acid combinations has also generated interesting results. When Ohta *et al* (1999) delivered the amino acid composition of an egg (at day 0 embryonation) into the yolk sac of a day 7 embryonated egg, chick hatch weight expressed as a ratio of initial egg weight, was significantly increased compared to untreated chicks. Bhanja and Mandal (2005) delivered combinations of various amino acids into the albumin of day 14 embryonated eggs. The most striking outcome of that work was the significant increases observed in humoral and cell-mediated immunity in chicks that had received the amino acid combinations of either lysine, methionine, cystine; threonine, glycine, serine or isoleucine, leucine, valine. Kadam *et al* (2008) also observed significantly improved humoral immunity, but not cell-mediated immunity, following the *in-ovo* administration of threonine only, into the yolk sac at day 14 embryonation. Threonine treated chicks were also heavier than control chicks at 4 weeks of age. The work of Bhanja *et al* (2004a) also identified the positive effect of *in-ovo* amino acid administration on chick weight.

The need for the chick to have immediate access to feed and nutrients at hatch to support gastric and intestinal development, nutrient and yolk digestion and absorption, immune function, muscle (especially breast muscle) deposition and chick growth, has been previously emphasized. With the foundation for each of these components occurring in the embryo and young chick, post-hatch nutrient supplements such as “Oasis hatchling supplement” (Dibner 1999) and “Chick-aide” (Suzuki *et al* 2008) have been designed. These are suitable for inclusion with hatchlings in either the hatching trays, or during transport to grower farms, to support early chick growth and development. Similarly, the identification of other substances, for example bioactive agents, that may enhance early chick performance, using similar methods of administration, drives further research in this area.

Given the protein content of MBM it is a suitable candidate for assessment of bioactive agents that may enhance early chick performance. Bioactive agents are also ideal candidates for early administration to the chick with the possibility of supporting and enhancing chick development, growth and general health. During late embryogenesis or the early post-hatch period, the main functions of the physiological systems are developing. Any increase in physiological function that occurs at this early stage has the potential to deliver continued benefits for the duration of the chicks' life. Therefore studies undertaken in this project evaluated the outcome, in terms of chick development, breast muscle weight and chick growth, of the delivery of MBM-derived substances following their administration *in-ovo* or to the chick during the early post-hatch period. The outcomes of this project have the potential to enhance chicken meat productivity with subsequent economic benefits, while also promoting a new and innovative use for MBM in the animal industries.

Project Objectives:

This project was designed to support and expand the use of MBM in the existing markets and to identify nutraceutical and nutritional properties of MBM that will further promote its inclusion in broiler diets. The aims of the project were to

1) identify bioactive peptides in MBM,

2) determine if these bioactive peptides have the capacity to improve the health, nutrition, and growth of broiler chickens,

3) evaluate the impact of the rendering process on the viability of MBM derived bioactive peptides, and

4) determine if dietary supplements (such as proteases) may enhance the bioavailability and or bioactivity of these peptides.

Methodology and Results

This project had two focus areas.

Focus area 1: **MBM protein and peptide extraction, fractionation and characterisation** The proteins/peptides present in MBM were identified, and the effect of the rendering conditions on the type and viability of the peptides present determined.

Focus area 2: **Chick evaluation of MBM peptides.** The ability of MBM-derived proteins and peptides to improve broiler chick production – with a focus on chick growth and breast muscle deposition, were assessed. These studies involved the delivery of the bioactive substances to the chick at the late embryonic stage *(in-ovo*) or during the first week post-hatch.

For the purposes of presenting the methodology and results used in each experiment, these focus areas are presented separately, with consecutive presentation of methodology and results, for ease of understanding, and to follow the flow of the research work.

Throughout both focus areas comparisons have been made between two samples of MBM, which have been identified as

1. MBM-1: From Rockdale Rendering facility (classed as a low temperature rendering facility)
2. MBM-2: From E.C. Throsby Pty Ltd (classed as a high temperature rendering facility).

At the outset of this project it was decided that comparison between two samples of MBM from different rendering plants would be the most appropriate way to represent the type of variation in peptides that could be expected from different rendering facilities in Australia.

As many aspects of each rendering facility vary, for example, the class and age of livestock slaughtered and therefore MBM raw materials and, the rendering process itself, differences between MBM from different facilities was expected. Analysis of MBM derived from identical raw materials rendered under the various rendering conditions utilised in Australia is the only way to accurately understand the impact of the rendering process on the MBM-derived peptide fragments. However from a practical perspective this is not feasible. Therefore the two samples used are examples only of the differences that may be seen in the protein/peptide content of MBM derived from rendering facilities using either high or low temperature rendering processes, while remembering that the raw materials used at each facility also vary.

Therefore, in this project, MBM sourced from two different rendering facilities has been used to provide an example of the variation in proteins/peptides that could be expected in Australian MBM. In addition, a proximate analysis and amino acid analysis of each sample has provided an indication of the composition of each of these batches of MBM.

Focus area 1. MBM protein and peptide extraction, fractionation and characterisation

1.1 Identifying peptides in MBM.

Initial laboratory analysis evaluated some of the basic properties of the protein/peptides extracted from MBM under a variety of conditions. The size range of peptides in both of the MBM samples was then investigated.

1.1.1 *MBM characterisation study 1*: To assess the water soluble protein/peptide extraction of MBM preparations:

Method:

100 mg of each of the MBM's were incubated overnight at room temperature, following which the pH and optical density (OD) at 280nm, of the supernatant was determined.

Results*:*

The pH and OD of the supernatant solution were;

	Neat	**1/10 dilution**	**1/100 dilution**
	pH	OD at 280nm	OD at 280
MBM-1:	7.3	5.44	0.44
MBM-2:	6.7	0.185	0.12

Note: under these conditions minimal protein was solubilized with the majority (~99%) remaining as fibrous and particulate insoluble material. There was visible lipid extracted into the soluble phase.

1.1.2 *MBM characterisation study 2*: Assess and compare the extraction of proteins in aqueous and detergent (ionic and non-ionic) environments using SDS-PAGE

Method:

Water and detergent soluble extractions of MBM proteins was undertaken by combining 500 mg of crude MBM and suspending it in 5 ml of Tris Buffered Saline with 10 mM EDTA and incubated for 1 hour at 29^{o}C – followed by overnight at 4^{o}C – then 2 hours at 30^{o}C.

Tris buffered saline (TBS)

TBS + 1% SDS (ionic (negative charge) detergent)

TBS + 1% Triton X-100 (non-ionic detergent)

TBS + 1% Tween-20 (non-ionic detergent)

TBS + 1% NP-40 (non-ionic detergent)

10 mM EDTA was included in each to suppress bacterial growth during the extraction period.

As a preliminary step, a small sample of the extracted material was first examined by SDS-PAGE. A more detailed and definitive analysis was then performed by water-soluble size fractionated MBM extracts by mass spectrometry.

For mass spectrometric analysis samples were passed through a micro-chromatography column (ZipTip), untreated or trypsinised (treated with 1/10 per wt dilution trypsin) and the bound peptide/ protein eluted with 80% Acetonitrile,

The Treatments studied were: MBM-

1 untreated soluble extracts MBM-1

trypsinised soluble extracts

MBM-2 untreated soluble extracts

MBM-1 trypsinised soluble extracts

Electrospray Mass Spectrometry was performed using the Biomolecular Mass Spectrometry Facility (BMSF) at the University of new South Wales.

Analysis of mass spectrographs were performed using Mascot software and the following search parameters:

- MS/MS ion Search by ESI-TRAP
- No enzyme
- Variable modifications; oxidation (M), Phospho (ST), Phospho (Y)
- Monoisotopic Mass Values
- Unrestricted Protein Mass
- +/- 6 ppm peptide mass tolerance
- +/- 0.6 Da Fragment Mass Tolerance
- 2 Missed Cleavages

From the resulting peptide fragments observed (ie > 5 amino acids in length), Mascot Searches with Probability Based Mowse Scoring identified the proteins listed below. The detailed analysis is presented in Appendix 1. The proteins and peptides identified in the test samples of MBM are presented in Appendix 2.

Results:

MBM-1: 337 queries

Y As expected overlapping peptide fragments matching **collagen type I alpha-1** of Bos Taurus (Cattle) (3 peptides),

Y And **collagen type I alpha-2** of Cattle (18 overlapping peptides) and Macaca mulatta (Rhesus Macaque)(14 overlapping peptides)

Y Also matching **Histone cluster 1.1/ 1.2/ 1.3/ 1.4** of Sus scrofa (pig), Mus Musculus (lab mouse), Homo sapiens, Canis lupus familiaris (dog), Equus calaballus (3 overlapping peptides)

Y **SnogY** of Streptomyces nogalater (an Actinobacteria)(2 overlapping peptides)

Y & additional- 300 unmatched peptides, *note: but as these have ions scores below the 66 level that define extensive homology identification further runs are necessary for solid validation. Suggested in these are a number of post-translational modifications (e.g. serine/ threonine/ tyrosine phosphorylation; methionine oxidation).

MBM-1 + Trypsin:

Y **Calmodulin** (5 peptides – 4 overlapping)

Y **AHNAK** neucleoprotein of rattus norvegicus (Rat) (2 overlapping peptides) also cattle, rat, cat, Monodelphis domestica (gray short tailed opossum)

Y & additional- 300 unmatched peptides, *but as these have ions scores below the 66 level that define extensive homology identification further runs are necessary for solid validation. Suggested in these are a number of post-translational modifications (e.g. serine/ threonine/ tyrosine phosphorylation; methionine oxidation).

MBM-2:

Y **Collagen type I alpha 2** of cattle (7 overlapping peptides) – same peptide matched in MBM-1 &

Y **beta-casein** of Bubalus bubalis (river buffalo)(2 non-overlapping peptides) – peptide of **CSN2 protein** (i.e. beta casein) of cattle also matched this.

Y & additional 51 unmatched peptides (28 of which appear to be post translationally modified i.e. 1-3 phosphates on Serine, threonine or tyrosine; or 1-3 oxidated on methionine).

MBM-2 + Trypsin:

Y **Collagen type I alpha 1** of cattle (3 peptides). (+ 3 trypsin peptides –auto catalysis)

Y & additional, 117 unmatched peptides.

Results from MBM characterisation section 1.1 Identify the peptides in MBM (study 1 and 2). These findings give positive identification of a number of MBM-derived proteins extracted by mild aqueous extraction (detailed in Appendix 2).

As expected collagen type 1 predominates in both MBM samples. In addition other proteins such as calmodulin, AHNAK nucleoprotein, histone, and beta-casein were novel protein identifications. Sequences of additional peptide fragments, were also identified. This was expected given the nature of MBM processing. These peptides may also have potential as bioactives, which was assessed in the chicken model. The results are presented in Focus area 2.

Alternative methods of extraction may also identify alternative peptides in MBM which may have bioactive properties.

1.2. Does the rendering process affect the peptides found in MBM?

1.2.1 MBM characterisation study 3: Comparison of peptides in MBM samples 1 and 2:

Method:

The peptides present in Rockdale (MBM-1) and Throsby MBM (MBM-2) samples were identified in soluble extracts obtained from each of these samples with and with-out trypsin fragmentation.

The samples studied were:

MBM-1 soluble extracts

MBM-1 trypsinised soluble extracts

MBM-2 soluble extracts

MBM-2 trypsinised soluble extracts

Results:

The identity of the resulting peptides was analysed by mass spectrometry, and is presented in Appendix 1. Details of the identified peptides are identified in Appendix 2. A brief summary of the main findings is presented below:

MBM-1:

Y As expected overlapping peptide fragments matching **collagen type I alpha-1** of Bos Taurus (Cattle) (3 peptides),

Y And **Collagen type I alpha-2** of Cattle (18 overlapping peptides) and Macaca mulatta (Rhesus Macaque)(14 overlapping peptides)

Y **Histone cluster 1.1/ 1.2/ 1.3/ 1.4**

Y **SnogY** of S. nogalater (2 overlapping peptides)
Y 300 additional peptides

MBM-1 + Trypsin:

Y **Calmodulin** (5 peptides – 4 overlapping)

Y **AHNAK** nucleoprotein (2 overlapping peptides)
Y 300 additional peptides

MBM-2:

Y **Collagen type I alpha 2** of cattle (7 overlapping peptides) – same peptide matched in MBM-1

Y **beta-casein** (2 non-overlapping peptides) – peptide of **CSN2 protein** (i.e. beta casein) of cattle also matched this.
Y 51 additional peptides

MBM-2 + Trypsin:

Y **Collagen type I alpha 1** of cattle (3 peptides). (+ 3 trypsin peptides –auto catalysis)
Y 117 additional peptides

Results from MBM characterisation study 3: Similarities and differences have been identified in the protein and peptide profiles obtained from these MBM samples.

MBM-1 contained collagen type I alpha-1 and alpha-2, Histone cluster, SnogY, calmodulin and AHNAK nucleoprotein.

MBM-2 contained collagen type I alpha-1 and alpha-2 and beta-casein.

All additional peptides observed in both MBM-1 and MBM-2 samples were comparable. Hence collagen type 1 alpha-1 and alpha-2 are present in both MBM-1and MBM-2. However, MBM-1 also contains Histone cluster, SnogY, calmodulin and AHNAK nucleoprotein, which were not present in MBM-2. In MBM-2 the only additional peptide not found in MBM-1 was beta casein.

1.2.2 MBM characterisation study 4: Proximate and amino acid analysis of MBM

Method:

Each MBM sample was analysed for dry matter, energy, nitrogen (protein), ash and lipid content following the standard methods of the Association of Official Agricultural Chemistry.

The amino acid content of both samples of MBM were determined with an LC-10A amino acid analyser (Shimadzu, Kyoto, Japan), using standard ion-exchange column chromatographic separation techniques with fluorimetric detection of amino acids after reaction with *o*-phthaldialdehyde (Li *et al*., 2006).

Results:

The outcomes of the proximate analysis are presented in Table 1.

Table 1: Proximate analysis

MBM source	**Dry Matter** (%)	**Energy** (MJ/kg)	**Protein** (%)	**Ash** (%)	**Lipid** (%)
Rockdale MBM-1	93.35	14.56	50.3	36.12	6.01
Throsby MBM-2	93.35	14.17	48.18	36.93	6.06

The dry matter content of both MBM-1 and MBM 2 is the same. However there is a higher percentage of ash and lipid and lower level of protein and energy in MBM-2 from the Throsby plant. This may also be indicative of the raw materials used for the MBM, with Throsby having a slightly higher proportion of bone-derived raw material. The Throsby plant typically processes older cows which are likely to have a higher level of bone than the younger livestock which are more routinely processed at Rockdale (Personal communication, Mr Bill Spooncer, Kurrajong Meat Technology Pty Ltd).

The amino acid levels in the MBM samples are presented in Table 2.

Table 2: Amino acid profile:

Amino Acid	**Rockdale (MBM-1) (mg/g)**	**Throsby (MBM-2) (mg/g)**
Aspartic acid	36.7	33.0
Threonine	18.1	16.3
Serine	22.4	21.5
Glutamic acid	64.0	57.8
Gylcine	77.9	74.0
Alanine	41.6	37.9
Valine	20.6	17.4
Methionine	8.0	6.7
Isoleucine	14.4	12.1
Leucine	30.5	26.3
Tyrosine	10.5	8.9
Phenilanine	16.1	14.0
Histidine	10.5	9.2
Lysine	27.3	23.4
Arginine.	37.8	34.8

MBM-2 from Throsby Pty Ltd had lower levels of all analysed amino acids compared to MBM-1 from the Rockdale plant. Of the crude protein levels, Rockdale MBM-1 contained approximately 87% protein nitrogen, and Throsby MBM-2 contained approximately 82% protein nitrogen. Hence Throsby MBM contained a greater level of non-protein nitrogen.

Results from MBM characterisation section 1.2 Does the rendering process affect the peptides present in MBM? (study 3 and 4)

Using MBM samples from Rockdale and E.C. Throsby rendering facilities for comparison it can be seen that fewer peptides are present in MBM-2 from the high temperature process (E.C. Throsby), compared to MBM-1 from Rockdale.

The proximate analysis indicated a lower level of energy and protein in the Throsby sample, and slightly higher levels of ash and lipid, compared to the low temperature facility at Rockdale.

Throsby MBM had lower levels of each of the analysed amino acids, and also a higher level of non-protein nitrogen.

Focus area 2. Chick evaluation

Two models for evaluating the potential for MBM-derived peptides to enhance chick performance through bioactivity were used in this project. The first model was the *in-ovo* delivery of the peptides, that is administration to the chick embryo at day 18 embryonation, 3 days prior to hatch. The second involved administration to the chick in the early post-hatch period, that is during the first 8 days after hatch.

All procedures were conducted in accordance with the NSW legislation and the general principles of the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes. The research work was supervised by the Animal Ethics Committee of the University of Sydney.

2.1 *In-ovo* delivery of MBM-derived peptides

Prior to *in-ovo* delivery of the MBM-derived peptides, a peptide or substance that enhanced aspects of early chick growth, which may include hatch weight, weekly chick weights, and breast weights, following its *in-ovo* delivery was identified. This active was then used as a positive control in subsequent experiments designed to assess bioactivity of MBM-derived peptides. The experiments used to identify a suitable positive control are outlined below.

2.1.1 Chick evaluation study 1. Establishing a positive standard for enhanced chick performance following its in-ovo delivery

Method:

Three experiments, identified as 0109, 0209 and 0309, each containing approximately 500 fertile eggs were designed to establish a positive standard for enhanced chick performance. A fourth experiment, 0110 was completed to repeat aspects of experiment 0309 to confirm the outcomes observed with the test substance glucose. Generally the materials and methods were similar in each study and so are presented together, however any differences between the experiments is noted.

Embryonated Eggs:

Day 0 Cobb line fertile eggs were obtained from a commercial supplier, Ingham's hatchery Casula. They were immediately set in the egg incubator (operating at 60% humidity; 99.5 F) with automatic turning of eggs every hour. At day 7 and 18 the eggs were candled and any infertile eggs were removed. At day 18 eggs were then randomly allocated to treatment groups (Appendix 3) and treatments administered.

Candidate Positive Bioactives:

Potential candidate bioactives for use as a positive control in the *in-ovo* model for assessing early chick-development and growth post-hatch were identified from current literature. Briefly, candidates include growth factors such as insulin-like growth factor-1 (IGF), insulin-like growth factor long R3 (IGF-1 long R3), epidermal growth factor (EGF), the amino acid threonine, a metabolite of the amino acid leucine, (β-hydroxy β-methylbutyrate (HMB)), glucose and whey protein. A brief overview of the main outcomes of relevant studies with each of these candidates are listed below

IGF- 1, when administered *in-ovo* at days 15 or 16 was observed by Kocamis *et al* (1998) and Kocamis *et al*., (1999) to enhance chick body weight, breast weight and feed conversion ratio.

IGF-1 Long R3 is an analogue of IGF-1 which has a chemically altered amino acid sequence, preventing binding to proteins. This increases its half-life, and therefore biological activity (personal communication, Professor Peter Wynn, Charles Sturt University).

EGF enhanced intestinal cell proliferation and weight gain following day 18 *in-ovo* delivery (Peebles, *et al*., 2001).

Threonine was administered into the yolk sac at 14 days embryonation, (Kadman *et al.,* 2008) with a subsequent increase in chick weight, feed conversion ratio and immune response.

HMB was delivered on day 17.5 embryonation by Tako *et al*., (2004) with subsequent increases in intestinal development both pre and post-hatch.

Glucose administration (Bhanja *et al*., 2008) at day 18 of embryonation, enhanced early intestinal weights and chick growth post hatch.

Whey protein has a very high component of edible proteins and amino acids. It is popular as a source of supplementary protein in human nutrition (Smithers, 2009). There are no published records of *in-ovo* administration of whey protein.

Peptide YY (PYY, a hormone of the gastrointestinal tract which is released in the presence of free fatty acids) had been considered as a candidate, however the cost of PYY deemed it impractical for *in-ovo* use.

Candidate Positive Bioactive Treatments (Delivery and Dosage).

Candidate bioactives were all delivered at day 18 embryonation. This time was chosen due to industry practice. On day 18 eggs in a commercial hatchery are handled for their transfer from the setting to hatching trays, and *in-ovo* vaccines are delivered. Therefore it is logical to use a similar time for *in-ovo* delivery of bioactives.

The delivery site for deposition of the candidates was either into the amnion (amn - fluid surrounding the chick, which is consumed (ingested) prior to hatch), into the chick (typically at the shoulder) or into the yolk sac (ys) (see Figure 1).

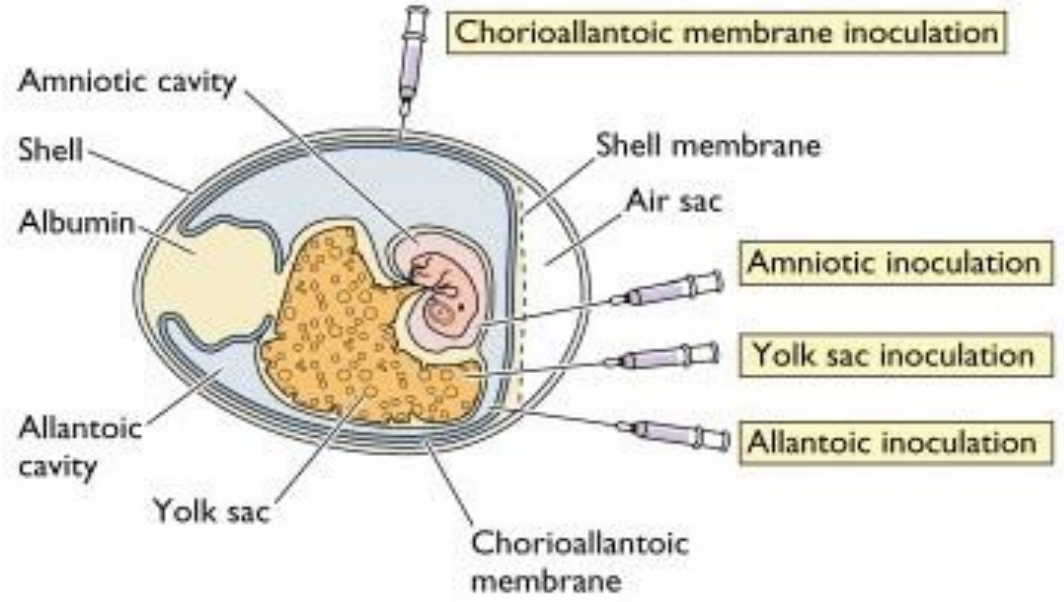

Figure 1: cutaway view of embryonated egg (Adapted from: Racaniello 2009).

Prior to delivery of candidate bioactives at day 18 embryonation, the egg was swabbed with 70% ethanol and the egg shell chipped at the point for entry of the needle. Following delivery, the egg shell was sealed with molten wax. Eggs were then moved into hatching trays and returned to the incubator.

A pilot study was run to compare combinations of the delivery point of needle through the egg shell with needle length to ensure accurate deposition of the bioactive at a pre-determined delivery site. The outcomes of this are presented in Table 3.

Table 3: Delivery site of Candidate Bioactives.

Delivery site	**Delivery point on egg shell**	**Needle length**
Amnion (amn)	Blunt end	1 inch
Chick (shoulder)	Blunt end	1.25 inch
Yolk sac (ys)	Side	1.25 inch.

For each of these delivery sites, the combinations outlined in Table 3 were used throughout experiments 0109, 0209, 0309 and 0110.

Treatment groups

The treatment groups for each experiment are outlined in Appendix 3.

Within Appendix 3 Treatments for Experiment 0109 are presented in Table 1, Experiment 0209 treatments in Table 2, Experiment 0309 in Table 3 and Experiment 0110 treatments are presented in Table 4. Experiment 0309 and 0110 were designed to increase sample numbers for each observation, as limited samples in the two initial experiments is likely to have reduced the strength of statistical analysis. In both of these experiments the delivery site for the active was the amnion as this is swallowed by the embryonic chick, with potential for direct effects on intestinal development and chick growth.

In each experiment the delivery vehicle/s used with any of the actives were also delivered alone to act as a vehicle control. A group of eggs which had their shell chipped, but no active inserted was included as a Controlhole only treatment, and finally there was a group that received no treatment which is identified as Control.

Observations:

Fertile embryos were weighed before placement in the incubator at day 0 embryonation. At hatch, individual chick weight and length (tip of the beak to the top of the toe nail) was measured. The birds were housed in cages in an environmentally controlled room, with temperature and lighting set to match the age requirements of the birds. The birds had free access to water and a wheat starter diet which was formulated to meet to National Research Council requirements for chicks of this age. Ten to twelve chicks were placed in each cage, with cages being allocated to treatments using a randomised block design. The blocking was intended to minimise any between cage/treatment effects of the environment within the room, for example, the proximity of heaters.

On days 4, 7, 14 (not in experiment 0109) and 21 post-hatch (days 6, 13 and 20 in experiment 0110) all chicks were weighed. On each of days 4, 7, (14 in experiment 0309 only) and 21 post-hatch a subsample of birds in each treatment group were selected at random. These birds were then euthanased and the following was collected:

- The breast (the premium cut of chicken meat) was removed from the chick carcase and weighed (not determined in experiment 0109).
- The length of each intestinal section (duodenum, jejunum, ileum and caeca) was measured and the weight of the total intestine (following removal of any digesta and the yolk sac) was recorded.
- Bird gender was determined.

All studies were terminated at day 21.

Statistical analysis:

Statistical analysis was performed by Professor Peter Thomson, Faculty of Veterinary Science, University of Sydney. Each analysis was conducted by fitting a linear mixed model to the data, implemented using a REML procedure in Genstat Rerelease 12.

Due to the increased variability of all measures over time (excluding hatch weights) data were log-transformed prior to analysis. The p-value was set at 0.05. Details of the analytical procedures are presented in Appendix 4. Following log-transformation back-transformed means were generated for each observation. These are presented in the relevant appendix for each study

Results:

In identifying a positive control for early chick growth, an ideal candidate would up-regulate several measurable parameters throughout the first 2-3 weeks post-hatch, compared with the control treatment (while also being mindful of comparisons with *in-ovo* administration of vehicle controls and control hole only treatments). The outcomes from each experiment are presented separately.

Experiment 0109: The main results for experiment 0109 are presented in Appendix 5 (App. 5). Table 1 outlines the summary of statistical analysis of all observations. At hatch chicks administered Long R3 IGF-1 *in-ovo* had a lower mean weight than the control chicks (Table 2, App. 5). This was the only statistically significant difference observed at hatch.

Comparison of mean body weight (Table 3, App. 5) illustrates varying outcomes for each treatment compared to the control groups throughout the study. Where statistically significant outcomes of treatment on mean body weight were observed, it was on one day (either day 4, 7 or 21) only, no ongoing impact was identified.

Mean gut weight and gut weight expressed as a percentage of body weight (%gut weight) of the treated birds was not statistically different from control birds (Table 4, App. 5).

Of the intestinal lengths, statistically significant effects of treatment were observed in the duodenum (Table 5, App. 5), jejunum and caecum (data not shown), however as with mean body weight, a statistically significant effect of treatment on sectional lengths was observed on individual days only (either day 4, 7 or 21), the effect was not repeatedly identified throughout the study.

Experiment 0209: Results from experiment 0209 are presented in Appendix 6 (App. 6). Table 1 presents the summary of the statistical analysis of all observations in experiment 0209.

Mean hatch weight of chicks in treatment groups were not statistically different from the control chicks (Table 2, App. 6). Similarly mean body weight of all treatment groups on each of days 4, 7, 14 and 21 post-hatch (Table 3, App. 6) were not different from mean body weight of the control chicks.

Mean breast weights are presented in Table 4, App. 4. On day 4 the mean breast weight of the control group was significantly higher than several treatment groups, including the vehicle control groups and various delivery and dose combinations for each active, being glucose, HMB and threonine. However on days 7 and 21 there were no statistically significant differences in mean breast weight between any treatment group and the control groups. The statistically significant observations seen on day 4 may have been due to the limited number of samples taken (5 in total) in each treatment group. Hence in experiment 0309 sample numbers were doubled to 10 observations/treatment group/sample day, increasing the accuracy of the assessment. When breast weight was expressed as a percentage of body weight, there were no statistically significant differences between any of the treatment groups.

Mean gut weight (Table 5, App. 6) demonstrated varying outcomes for each treatment compared to the control groups throughout the study. Where statistically significant outcomes of treatment on gut weight were observed, it was on one day (either day 4, 7 or 21) only, no ongoing impact throughout the study was identified. Percentage gut weight was not altered to a statistically significant level in treated chicks compared to the control chicks.

There were no significant effects of *in-ovo* treatments on the length of intestinal sections; duodenum (Table 6, App. 6) jejunum, ileum and caecum.

Experiment 0309: Results from experiment 0309 are presented in Appendix 7 (App. 7). Table 1 outlines the summary of the statistical analysis of all observations in experiment 0309. There was no difference between mean hatch weights of treatment chicks compared with control chicks (Table 2, App.7).

The only statistically significant difference in mean body weights throughout the experiment was observed on day 7, when Glucose 50mg treated chicks had a higher mean weight compared with the control DDW chicks (Table 3, App. 7). However, Glucose 50mg treated chicks demonstrated a clear trend during the first 2 weeks of growth, with higher mean body weights than all other groups on days 4, 7 (Figure 1, App. 7) and Day 14 (Figure 2 and Table 3 App. 7). Overall the Glucose 50mg treated chicks had a 5% increase (not statistically significant) in mean body weight compared with the control chicks.

Mean breast weights were influenced by treatment, with Glucose 50 mg treated chicks demonstrating a higher mean breast weight on days 4, 7 and 14, compared with the control chicks (Table 4, App. 7). Chick gender influenced this outcome with male chicks of the glucose 50 mg treatment group having statistically significant higher breast weights compared to males of the control group throughout the study (Figure 3. App. 7). Overall the

Glucose 50mg treated male chicks had a 9% increase ($P<0.05$) in mean breast weight compared with the male chicks of the control group.

Mean gut weight of Glucose 50 mg treated chicks were significantly higher than gut weight of control chicks which was especially evident on days 4, 7 and 21 post-hatch (Table 5, App. 7). Overall mean gut weights of Glucose 50 mg *in-ovo* treated chicks was 8% higher than the control chicks ($P<0.05$).

The effects of *in-ovo* treatment on intestinal length (Table 6, App. 7) was evident in the jejunum of Glucose 50mg treated chicks, which on average had longer jejunum (a 4% increase) compared to the control chicks ($P<0.05$).

Experiment 0110: Results from Experiment 0110 are presented in Appendix 8 (App.8). Table 1 outlines the summary of the statistical analysis of all observations in experiment 0110. There was no difference between mean hatch weights of treatment chicks compared with control chicks (Table 2, App.8).

There was no statistically significant effect of any of the *in-ovo* treatments, including delivery of glucose, on mean body weight during the experiment (Table 3, App. 8). However, overall there was a notable trend for Glucose treated birds to have higher mean body weights, compared to the control birds. This represented an average increase of 4% in body weight (not statistically significant) throughout the study.

The *in-ovo* administration of 50mg Glucose did generate a trend for increased breast weight, with an overall increase in mean breast weight of 9% (not statistically significant). Throughout the study the *in-ovo* treatments did not generate any significant effects on mean breast weight (Table 4, App. 8).

In-ovo treatment with glucose 50mg increased the percentage of breast weight (that is breast weight as a percentage of live chick weight) by 4.4% ($P<0.05$) compared to the control chicks on day 7 (Table 5, App 8). Further, chick gender influenced this outcome with male chicks of the glucose treatment group having higher percentage breast weight throughout the experiment, compared to the males of the control group.

The *in-ovo* administration of 50mg Glucose had no statistically significant effects on mean gut weight compared to untreated control chicks (Table 6, App. 8). Occasional differences of statistical significance were observed between groups, for example on day 4 the mean gut weight of the glucose treated chicks was significantly higher than the saline treated chicks, but no clear trends were evident. Similarly in terms of the mean length of the segments of the chicken intestine (duodenum, jejunum, ileum and caecum), there were no statistically significant differences between Glucose treated chicks and the control chicks.

Results from *Chick evaluation study 1. Establishing a positive standard for enhanced chick performance following its in-ovo delivery*

Outcomes from these experiments indicate that *in-ovo* administration of Glucose 50mg into the amnion is effective at enhancing several components, including body weight, breast and gut weights of early chick growth. While these observations were clearly evident in experiment 0309, it was not so in 0209. However, the comparatively low numbers of samples taken on each observation day in 0209 is likely to have limited identification of

these effects. In experiment 0309 sample numbers were doubled on each observation day which enabled clear identification of Glucose-50 induced upregulation of early chick growth. Experiment 0110 reconfirmed the ability of *in-ovo* administration of 50mg Glucose into the amnion to enhance several components of early chick growth including body weight and breast weight.

We recommend that *in-ovo* administration of 50mg of glucose be utilised as the positive control for *in-ovo* assessment of MBM-derived peptides on early chick development. Parameters to be measured for identification of early chick growth and development include chick weight, breast and gut weights, and intestinal lengths, throughout the first 3 weeks post-hatch.

2.1.2 Chick evaluation study 2. In-ovo delivery of MBM-derived peptides

In this series of experiments two *in-vivo* studies (identified as experiment 0210 and 0310) were designed to assess the impact of MBM-derived peptide fractions on early chick growth following their *in-ovo* delivery. The protein/peptide fractions were isolated from two different sources of MBM, Rockdale MBM and E.C. Throsby MBM. Experiment 0210, included fractions of proteins and peptides isolated from Rockdale MBM, and Experiment 0310, included proteins and peptide fractions isolated from Rockdale MBM and E.C. Throsby MBM. As highlighted previously using these two sources of MBM is designed to provide an indication of the differences that may be seen in the bioactivity of the protein and peptides isolated from rendering facilities using either high temperature (E.C. Throsby Pty Ltd) or low temperature (Rockdale) rendering processes.

Prior to the chick experimentation, peptides and protein fractions were isolated from the raw MBM samples. The final outcome of this process was the retrieval of peptides and proteins of pre-determined molecular weight cut-offs, with a final purification step, prior to their *in-ovo* delivery. The methods used for the peptide preparation and chick experimentation are presented below.

Method:

Preparation of peptide/protein fractions.

MBM obtained from Rockdale and E.C. Throsby Pty Ltd were the source material for protein and peptide fractions administered to the chick *in-ovo*. Each extract was fractionated by size exclusion centrifugation, using commercial, Amicon (Millipore) centrifugal Filter devices, that deploy regenerated cellulose filters of defined molecular weight cut-offs. This enables the separation of proteins and peptides of specific size ranges, permitting the purification of low molecular weight peptides and the enrichment of proteins and protein fragments. For Rockdale MBM, three size ranges of proteins/peptide fractions were prepared; <3kDa, 3-100kDa and >100kDa. With the Throsby MBM material the protein preparations were divided into either <50kDa or >50kDa fragments. The detailed method outline for each preparation is presented in Appendix 9. Rockdale is presented in Figure 1 and E.C. Throsby in Figure 2.

In brief a crude supernatant of MBM was initially prepared following several sequential suspension (in phosphate buffered saline, (PBS)) and centrifugation steps. The supernatant

was then subjected to filtration under vacuum, through a 0.45 um filter initially and then a 0.22um filter. The eluate was then filtered to provide fractions of the specified size range for each specific source of MBM. Sterility of each preparation for *in-ovo* treatment was assured by 0.22 um filtration. Subsamples of the different fractions were taken for quantitative analysis of protein concentration using a commercial (Biorad) kit and analysis of peptide absorbance at 206, 212 and 230 nm, and qualitatively by reverse-phase HPLC profiling.

Chick experimentation.

Generally the materials and methods of the chick experiments are identical to those presented in Chick evaluation study 1. Brief details, including any variations in the methods, from chick evaluation study 1, are outlined below.

Embryonated eggs:

500 Day 0 Cobb fertile eggs were obtained from Inghams hatchery, Casula, for both Experiments 0210 and 0310.

Treatments

The treatment groups for each experiment are outlined in Appendix 10. Here the treatment groups for Experiment 0210 are in Table 1 and Experiment 0310 treatments are presented in Table 2.

Candidate peptides/protein fractions were all delivered at day 18 embryonation. The delivery site for deposition of the candidates was into the amnion, the fluid surrounding the chick, which is consumed (ingested) prior to hatch. In each experiment the delivery vehicle/s used with any of the actives were also delivered alone to act as a vehicle control. A group of eggs which had their shell chipped, but no active administered were included as a Controlhole-only treatment. Finally there was a group that received no treatment and this was identified as Control.

***In-ovo* administration (Delivery and Dosage)**

At day 18, any infertile eggs were identified by candling and removed from the experiment. Fertile eggs were then randomly allocated to treatment groups (Appendix 10) and treatments administered.

Prior to delivery of candidate bioactives at day 18 embryonation, the egg shell was swabbed with 70% ethanol and the egg shell chipped at the point for entry of the needle. Following delivery, the egg shell was sealed with molten wax. Eggs were then moved into hatching trays and returned to the incubator.

Observations:

On day 0 embryonation all eggs were weighed prior to being placed in the incubator. At hatch, individual chick weight and length was measured. On days 6, 13 and 20 post-hatch all chicks were weighed.

On each of observation days 4, 7, 14 and 21 post-hatch a subsample of birds were chosen at random from each treatment group. These birds were weighed prior to being euthanased. The following was determined/collected from each of these birds:

- The breast was removed from the chick carcase and weighed.
- The length of each intestinal section (duodenum, jejunum, ileum and caeca) was measured and the weight of the total intestine (following removal of any digesta and the yolk sac) was recorded.
- Bird gender was determined.

Each study was terminated at day 21.

Statistical analysis:

Statistical analysis was performed by Associate Professor Peter Thomson, Faculty of Veterinary Science, University of Sydney. Each analysis was conducted by fitting a linear mixed model to the data, implemented using a REML procedure in GenStat Rerelease 12, as previously described.

Results:

Peptide/protein fractions for Experiments 0210 and 0310:

The concentration of protein in each preparation in Experiment 0210 and 0310 are presented in Appendix 10, Tables 1 and 2 respectively. Protein concentration varied from 0.1mg/500ul with Fraction 5 in Experiment 0310, to 0.5mg/500ul in Fraction 7 in Experiment 0310. Note that the extraction of the fractions from the raw MBM material for each experiment was completed separately.

Experiment 0210:

The outcomes from Experiment 0210 are presented in Appendix 11 (App. 11). Table 1 outlines the summary of the statistical analysis of all observations in Experiment 0210. Chick hatchability was not significantly affected by the administration of any of the candidate bioactives (data not shown). There was no difference between mean hatch weight (Table 2, App.11) and hatch length of treatment chicks compared with control chicks.

There were no statistically significant differences in mean body weight based on treatment groups on each observation day throughout the experiment (Table 3, App 11).

Treatment did not affect breast weight (Table 4, App 11) however it did influence percentage breast weight (Table 5 App. 11). A reduction in percentage breast weight was observed on day 7 with the chicks receiving peptide fractions 1, 2 and 3 *in-ovo* and, in the control chicks where a hole was chipped into the shell.

Gut weight (Table 6 App 11) was not affected by the fraction treatments, however on day 4 the vehicle (PBS) control treated chicks recorded a notable (P=0.067) reduction in average gut weight.

There no statistically significant differences were observed in the mean length of the intestinal segments duodenum, jejunum, ileum and caecum, between treatments and the control groups.

Throughout Experiment 0210 there was no evidence of enhanced early chick growth and development following *in-ovo* administration of the positive control (50 mg Glucose). This

was unexpected given the outcomes of Experiments 0309 and 0110, as described under the section Chick evaluation study 1. *Establishing a positive standard for enhanced chick performance following its in-ovo delivery*. In these experiments glucose was seen to enhance aspects of early chick growth. As the positive control did not deliver the expected outcomes in Experiment 0210, this also places a question over the outcomes observed in chicks treated with the peptide fractions. Therefore a further study was performed (Experiment 0310) which duplicated the *in-ovo* delivery of fractions 1-3 derived from Rockdale MBM (though final dose rates varied with Rockdale >100kDa from Experiment 0210 and so for clarity the extracted fraction treatments are identified as fractions 5-7 in Experiment 0310, – see Tables1 and 2, Appendix 10). Finally Experiment 0310 also included two peptide fraction preparations from E.C. Throsby (Table 2, Appendix 10).

Experiment 0310

The outcomes from Experiment 0310 are presented in Appendix 12 (App.12). Table 1 outlines the summary of the statistical analysis of all observations in Experiment 0310. Chick hatchability was not significantly affected by the administration of any of the candidate bioactives (data not shown).

Mean body weights, at hatch and for the following 3 weeks of age were not significantly altered by peptide treatments (Table 2, App 12). Interestingly the *in-ovo* administration of PBS alone in this study did significantly reduce mean chick weights at day 7, 14 and 21 compared to the birds receiving fraction 9 (Throsby >50kDa), at day 7 and 14 compared to birds receiving fraction 8 (Throsby <50kDa) and at day 7 compared to birds receiving fraction 7 (Rockdale .>100kDa) and glucose.

Mean breast weights (Table 3, App 12) and mean percentage breast weights (data not shown) of chicks receiving the peptide fractions *in-ovo* were not significantly different from those of the control chicks.

Similarly gut weight (Table 4, App12) was not significantly affected by the peptide treatment, and nor was the mean length of the intestinal segments duodenum, jejunum, ileum and caecum, compared to the control chicks.

As with Experiment 0210, throughout Experiment 0310 the administration of the positive control (50mg Glucose) did not generate a significant up-regulation of chick development or growth on days 4, 7, 14 and 21 of age. This is in contrast to two previous experiments (Experiment 0309 and 0110) but similar to Experiment 0210. The reasons for this lack of response are not known but possible influences are presented in the discussion.

Results from Chick evaluation study 2. In-ovo delivery of MBM-derived peptides

Nine peptide/protein fraction isolates have been delivered *in-ovo* to the chick embryo, with subsequent evaluation of chick performance following hatch. Early chick growth and development was not found to be enhanced following the *in-ovo* delivery of these fractions. However these outcomes were observed in experiments where the chosen positive control did not enhance early chick growth. The significance of this for interpretation of the results following treatment with the MBM-derived peptide fractions is debated in the discussion.

2.2 Delivery of MBM-derived peptides to the chick post-hatch.

The administration of bioactive peptides to chicks during the early post-hatch period also represents an opportunity for the peptides to enhance early post-hatch development and growth, that may translate in to improved performance throughout the production cycle. This was assessed in meat chicks grown to 30 days of age.

In preparation for the chick experimentation, peptides and protein fractions were isolated from the raw MBM samples, as described in study 2, section 2.1.2. The final outcome of this process was the retrieval of peptides and proteins of pre-determined molecular weight cut-offs, which are detailed in Appendix 14.

2.2.1 Chick evaluation study 3: Delivery of MBM-derived peptides to day old chicks

Two chick experiments were completed to evaluate the effect of MBM-derived peptides on early chick growth, following their oral delivery during the first week post-hatch. These experiments are identified as experiment 0410 and 0610. Experiment 0410 included treatment of Cobb chicks (which have been used in all previous studies, as the most commonly grown line of commercial broiler in the Australia poultry industry) with peptide fractions isolated from both the Rockdale MBM-1 and E.C. Throsby MBM-2.

Experiment 0610 was completed using the Ross line of broiler chick as some reports suggest that this line of broiler may be more protein sensitive than the Cobb bird. In Experiment 0610 we evaluated the peptide fractions from Rockdale further, which included the subdivision of the 3-100kDa group, into two smaller size ranges of 3-30kDa and 30-100kDa.

The methods used in both studies were similar and are presented together, with any differences being highlighted. The results of each experiment are presented separately.

Methods

Preparation of peptide/protein fractions. This procedure is similar to that described under *section 2.1.2 Chick evaluation study 2. In-ovo delivery of MBM-derived peptides*. This procedure was completed separately for each experiment.

For Rockdale MBM, five size ranges of proteins/peptide fractions were prepared; <3kDa, 3-100kDa, 3-30kDa, 30-100kDa and >100kDa. With the Throsby MBM material the protein preparations were divided into either <50kDa or >50kDa fragments. A detailed outline of the extraction method that was used and common for both treatments is presented in Appendix

13, Figure 1. The two MBM samples underwent different fractionation procedures and these are presented for Rockdale MBM-1 in Figure 2, and for Throsby MBM-2 in Figure 3 of Appendix 13. The sterility of treatments was ensured by 0.22 um filtration. As with previous samples, subsamples of each fraction preparation were taken for analysis of protein concentration.

Chick experimentation.

Day old chicks:

For Experiment 0410 day old male Cobb chicks and for Experiment 0610 day old male Ross chicks were obtained from commercial hatcheries. Chicks were immediately transported to the Poultry Unit, University of Sydney, Camden, randomly allocated to treatments in groups of 10 and placed in their pens with free access to feed and water.

Treatments and peptide administration,

The treatment groups for each experiment are outlined in Appendix 14, with the treatment groups for Experiment 0410 presented in Table 1, Experiment 0610 treatments in Table 2.

In both studies peptides/protein fractions were all delivered on four occasions starting at day old, with subsequent deliveries on day 3, 5 and day 8. 250ul of peptide fraction was delivered on each occasion using a blunt ended tip on a multi-stepper pipette. Each chick received a total of 1ml of the peptide fraction preparation over the four days of administration. In each experiment the delivery vehicle/s used with any of the actives were also delivered alone to act as a vehicle control. In experiment 0410 a treatment group of handling the chicks was included – to identify any effect that handling the chicks for the treatments may have on their growth and performance. In both experiments there was a group that received no treatment which was identified as Control.

The amount of peptide/protein administered to each chick in each fraction is also presented in Appendix 14.

Observations:

On day one, at the start of each experiment each chick was identified with a uniquely numbered wing tag and then weighed.

On days 8, 15, 22 and 29 post-hatch all chicks were weighed.

On each of observation days 10, 16, 23 and 30 a subsample of birds was chosen at random from each treatment group. These birds were weighed prior to being euthanased. The following was determined/collected from each of these birds:

- The breast was removed from the chick carcase and weighed.
- The length of each intestinal section (duodenum, jejunum, ileum and caeca) was measured and the weight of the total intestine (following removal of any digesta and the yolk sac) was recorded.

- Bird gender was determined.

Each study was terminated at day 30.

Statistical analysis:

Statistical analysis for these experiments was as previously described (Appendix 4).

Results:

Protein concentration of peptide/protein fractions for Experiments 0410 and 0610:

The protein concentrations of the preparations used in these experiments are presented in Appendix 14. In Experiment 0410 (Appendix 14, Table 1) protein concentration ranged from 0.5mg/ml for Fraction 6 (Rockdale 3-100kDa) to 0.8mg in Fraction 7 (Rockdale >100kDa). In Experiment 0610 both Fractions 11 and 12 (Rockdale 3-30 and 30-100kDa) contained <0.5mg/ml (which equates to the total amount received by each chick), and Fraction 15 (Rockdale all peptides) had the highest quantity of protein, being 2.2mg/ml (Appendix 14, Table 2).

Experiment 0410: The results from Experiment 0410 are presented in Appendix 15 (App. 15). Table 1 outlines the summary of the statistical analysis of all observations in the experiment. Birds that were handled without any treatment administration were not significantly different from the control chickens in all of the recorded observations.

There was no statistically significant effect of any of the peptide treatments on mean body weight during the experiment (Table 2, App. 15). However, overall there was a notable trend, with at least 80% of the birds administered Fraction 6 (Rockdale 3-100kDa) weighing more than the mean body weight of the control birds. These birds demonstrated an average increase of 9.6, 7.7% and 6.8% in mean body weight (not statistically significant) on days 15, 22 and 29 respectively of the experiment compared to the control birds (Figures 1 and 2, App 15).

The administration of Fraction 6 (Rockdale 3-100kDa) also generated a trend for increased breast weights in at least 70% of treated birds compared to the control birds. Overall the mean breast weights were 13% and 9% respectively higher (not statistically significant), than the control birds on day 16 and 30 respectively (Figure 3, App 15). Throughout the study there were no statistically significant effects of any of the peptide fraction treatments on mean breast weight (Table 3, App. 15), nor mean percentage breast weight (Table 4, App 15).

There were no significant effects of treatment with any of the peptide fractions on gut weights (Table 5, App 15), percentage gut weights nor any of the intestinal lengths (duodenum, jejunum, ileum and caeca) compared to untreated control chicks.

Given the increased mean body and breast weights observed in Experiment 0410 with peptide Fraction 2, Rockdale 3-100kDa compared to the control chicks, a closer examination of this fraction was designed for Experiment 0610. This involved the use of an alternative line of broiler bird (Ross line) which may be more sensitive to dietary protein, and the subdivision of Rockdale fraction of 3-100kDa into two additional fractions, consisting of size

ranges 3-30kDa and 30-100kDa, to more closely examine the potential effects of the peptides from within the larger molecular weight size range on chick performance.

Experiment 0610:

The outcomes from Experiment 0610 are presented in Appendix 16 (App. 16). Table 1 outlines the summary of the statistical analysis of all observations in Experiment 0610.

Unfortunately the batch of birds placed in this experiment was affected by an Infectious Stunting Syndrome. The chicks were slightly lighter than expected on arrival at the Poultry unit – averaging 37-38 grams, where ideally day-old chicks should weigh at least 40 grams. By day 12 there were undersize chicks noted in some pens (which were evenly distributed across the treatment groups) which were not able to compete with their pen mates. These chicks were euthanased and Poultry Veterinarian Dr Peter Groves performed a post-mortem on each chick. The birds were noted to be "suffering from serious problems with digestion, possibly associated with Infectious stunting syndrome. This is a viral infection which is poorly understood, and caused by a group of viruses..... It can be the result of vertical transmission and commonly seen in chicks from young breeder flocks. There is no treatment and euthanasia of affected birds is required. Infectious stunting syndrome is also present in commercial flocks at this time"(personal communication from Dr Peter Groves, 4th November 2011). Once all of the visibly affected birds were removed, the remaining birds performed more in line with normal expectations. However, the incidence of this syndrome may have confounded the outcomes of this study, and would appear to have impacted on overall bird performance for at least the first two and a half weeks of the study. Hence results up to and including day 15 should be interpreted with extreme caution. Therefore the Day 22 and Day 30 outcomes are the focus of the results presented in this report on Experiment 0610.

Overall peptide treatments did not have any significant effect on body weight (Table 2, App 16) however with Fractions 11 (Rockdale 3-30kDa) and 13 (Rockdale 3-100kDa) there was a notable increase of 7 and 9% respectively in mean body weight compared to the untreated control birds on day 22 (Figure 1, App 16). At least 70% of birds treated with these peptide fractions demonstrated an increase in body weight compared to the mean of the control birds.

Mean breast weight (Table 3, App 16) was not significantly influenced by peptide treatments, however on day 23 Fractions 11 (Rockdale 3-30kDa) and Fraction 15 (Rockdale all peptides) both demonstrated a 9% increase in breast weights compared to the control birds. On day 30 a 16% increase (not significant) was also observed with Fraction 13 (Rockdale 3-100kDa) (Figure 2, App 16). Again, at least 70% of the birds receiving these peptide fractions demonstrated an increase in breast weight compared to the mean of the control birds. Percentage breast weight was not significantly altered in relation to treatment with peptide fractions (Table 4, App 16).

There was a significant effect of peptide and PBS treatment on gut weights on day 30 ($p=0.002$) (Table 1, App 16), however as the Control-PBS treatment also had a higher average gut weight compared to the untreated control chicks at this time, this effect cannot be directly related to the peptide fraction treatment (Table 5, App 16).

Interestingly however are the lengths of the jejunum and ileum in the peptide treated chicks. (Tables 6 and 7 respectively, App 16). On day 30, Fraction 13 (Rockdale 3-100kDa), Fraction 14 (Rockdale >100kDa) and Fraction 15 (Rockdale all peptides) had significantly ($P<0.05$) longer jejunum (approximately 13%) than the untreated control birds. These same treatments generated significantly ($P=0.015$) longer ileum at day 30, as did Fraction 11 (Rockdale 3-30kDa), (Fraction 15 generating the highest increase of 25% in ileal length) compared to the untreated birds (Figure 3, App 16).

The significant effect of the treatments on caecal length were generally from observations on day 16, which as indicated previously need to be treated with caution (in this study the peptide treated chicks (Fraction 10 – Rockdale <3kDa, Fraction 11-Rockdale 3-30kDa and Fraction 13- Rockdale 3-100kDa) had significantly shorter caecal lengths compared with the control chicks at this time). However on day 30 Fraction 14, (Rockdale >100kDa) had mean caecal lengths that were significantly longer than the untreated control chicks.

While interpreting results from Experiment 0610 with caution, birds receiving Fraction 11 (Rockdale 3-30kDa) and Fraction 13 (3-100kDa) have demonstrated the more frequent improvement in chick performance. This is similar to observations from Experiment 0410 where Fraction 6, Rockdale 3-100kDa demonstrated a regular increase (not statistically significant) in chick weights and breast yield. These most promising peptide fractions will also be evaluated in the final stage of the project, when assessing the effects of adding protease to the peptide fractions, as opposed to administering the peptide fractions alone, on chick performance.

Results from Chick evaluation study 3: Delivery of MBM-derived peptides to day old chicks

From experiments 0410 and 0610 the peptide fraction which demonstrated the greatest improvement in chick performance (especially body and breast weights) was Rockdale 3-100kDa. This fraction was divided into two size ranges (Rockdale 3-30kDa and 30-100kDa) for testing and comparison with the Rockdale 3-100kDa treatment in Experiment 0610. The fraction containing the smaller molecular weight size range of 3-30kDa demonstrated increased performance (but was not always statistically significant) similar to that observed in the 3-100kDa treated chicks run concurrently in Experiment 0610.

2.2.2 Chick evaluation study 4: Delivery of MBM-derived peptides to day old chicks following peptide treatment with a protease.

While enhanced chick performance has been observed, in terms of increased chick body and breast weights, with Rockdale 3-100kDa size range of peptides, pre-treatment of the peptide fractions with a protease may release other peptides from the parent protein/peptide fractions, and these peptides may also possess bioactivity. To assess this, we used a protease with keratolytic activity that has been shown to demonstrate improved growth when incorporated in to broiler diets (Odetallah *et al* 2003; Wang *et al* 2006). The inclusion rate of the protease in the peptide fraction was calculated to match its recommended inclusion rate in a poultry diet, based on protein concentration, which is 0.5kg/tonne.

Two chick experiments were completed to evaluate the effect of the pre-treatment of MBM-derived peptide fractions with protease, on early chick growth, following their oral delivery during the first week post-hatch. These experiments are identified as experiment 0111 and 0211. In both experiments Cobb male chicks were administered peptide fractions with or without pre-treatment with a protease immediately prior to their oral administration to the chick. In both of these studies a crude peptide preparation of each MBM was also delivered. This was designed to assess whether the combined peptides fraction may also demonstrate bioactivity.

In experiment 0111 peptides were derived from Throsby MBM. They were the fractions: <50kDa, >50kDa and a crude peptide preparation. In experiment 0211 MBM-peptide fractions were derived from the Rockdale MBM and they were the fractions 3-100kDa, >100kDa and crude peptides. The methods used in both studies were similar and are presented together, with any differences being highlighted. The results of each experiment are presented separately.

Methods

Preparation of peptide/protein fractions. This procedure is similar to that described under *section 2.1.2 Chick evaluation study 2. In-ovo delivery of MBM-derived peptides*. This procedure was completed separately for each experiment.

For Rockdale MBM, three size ranges of proteins/peptide fractions were prepared; crude peptides, 3-100kDa, and >100kDa. With the Throsby MBM material the protein preparations were divided into crude peptides, <50kDa or >50kDa fragments. A detailed outline of the extraction method that was used and common for both treatments is presented in Appendix 13, Figure 1. The two MBM samples underwent different fractionation procedures and these are presented for Rockdale MBM-1 in Figure 2, and for Throsby MBM-2 in Figure 3 of Appendix 13. The sterility of treatments was assured by 0.22 um filtration. As with previous samples, subsamples of each fraction preparation were taken for analysis of protein concentration.

Chick experimentation.

Day old chicks: Day old male Cobb chicks were obtained from a commercial hatchery. Chicks were immediately transported to the Poultry Unit, University of Sydney, Camden, randomly allocated to treatments in groups of 10 and placed in their pens, which had been randomly assigned to treatments, with free access to feed and water.

Treatments, protease treatment of peptides and peptide administration,

The treatment groups for each experiment are outlined in Appendix 17, with the treatment groups for Experiment 0111 presented in Table 1, Experiment 0211 treatments in Table 2.

In both studies peptides/protein fractions were all delivered on four occasions starting at day old, with subsequent deliveries on days 3, 5 and 8 of age. 250ul of peptide fraction was delivered on each occasion using a blunt ended tip on a multi-stepper pipette. Each chick received a total of 1ml of the peptide fraction preparation over the four days of

administration. In each experiment the delivery vehicle/s used with any of the actives were also delivered alone to act as a vehicle control.

Peptide fractions pre-treated with the protease received the equivalent of 0.5kg/ton protein of the protease, which was mixed into the peptide preparation and allowed to incubate at room temperature for 5 minutes. The reaction was then stopped by placing the tube in ice. The peptide preparation was then delivered to the first chicks of that treatment group within 5 minutes of the reaction been stopped. The time it took to administer the peptide treatments to each cage of 10 chickens, and all 5 cages of chickens from the one treatment group was recorded. On average it took 8 minutes to deliver the peptides to one treatment group of birds (50 birds in total, in 5 cages of 10 birds each).

The amount of peptide/protein administered to each chick in each fraction is also presented in Appendix 17.

Observations:

On day one, at the start of each experiment each chick was identified with a uniquely numbered wing tag and then weighed. The cages were allocated to treatments using a completely randomised design, without blocking, had had been applied in all previous experiments. Cages were not blocked in these studies as there had been no significant effect of the blocks in all previous experiments and the use of blocks reduces the degrees of freedom in an analysis.

On days 8, 15, 22 and 29 post-hatch all chicks were weighed.

On each of observation days 10, 16, 23 and 30 a subsample of birds was chosen at random from each treatment group. These birds were weighed prior to being euthanased. The following was determined/collected from each of these birds:

- The breast was removed from the chick carcase and weighed.
- The length of each intestinal section (duodenum, jejunum, ileum and caeca) was measured and the weight of the total intestine (following removal of any digesta and the yolk sac) was recorded.
- Bird gender was determined.

Each study was terminated at day 30.

Statistical analysis:

Statistical analysis for these experiments was the same as previously described (Appendix 4).

Results:

Protein concentration of peptide/protein fractions for Experiments 0111 and 0211:

The protein concentrations of each preparation used in these experiments is presented in Appendix 17. In Experiment 0111 (Appendix 17, Table 1) protein concentration ranged from 0.6mg/ml for Fraction 16 (Throsby <50kDa) to 2.2mg/ml in Fraction 18 (Throsby Crude peptides). In Experiment 0211 protein concentration in the sub-fractions ranged from

0.5mg/ml in Fraction 19 (Rockdale 3-100kDa) and Fraction 20 (Rockdale >100kDa) to 1.3mg/ml in the Rockdale Crude peptide fraction (Fraction 21) (Appendix 17, Table 2).

Experiment 0111: The results from Experiment 0111 are presented in Appendix 18 (App. 18). Table 1 outlines the summary of the statistical analysis of all observations in the experiment.

There was no statistically significant effect of the Throsby peptide treatments with or without protease treatment, on mean body weights throughout the experiment (Appendix 18, Table 2). Further, breast weights (App. 18, Table 3), percentage breast weights (App. 18, Table 4), gut weights (App.19 Table 5), percentage gut weights, and the length of any of the intestinal segments (data not shown) were not significantly altered by the peptide treatments with and without protease, when compared to the control birds.

Experiment 0211:

The outcomes from Experiment 0211 are presented in Appendix 19 (App. 19). Table 1 outlines the summary of the statistical analysis of all observations in Experiment 0211.

There was no statistically significant effect of the Rockdale peptide treatments on the mean bird body weights throughout the experiment (App 19, Table 2).

Mean breast weight was not significantly affected by the peptides (with and without protease treatment) (App 19, Table 3) however on day 23 the percentage of breast weight (App. 19, Table 4) was significantly increased ($p=0.05$) (by 12.5%) in birds administered Fractions 19 (Rockdale 3-100kDa) and Fraction 19 + protease, and by 8.5% in chicks administered Fraction 20 + P (Rockdale >100kDa with protease) (Figure 1, App 19). On day 10, birds that had received the Rockdale crude peptide (Fraction 21) with protease, had a significantly lower percentage mean breast weight (by 7.8%) compared with the control chicks. No other significant effects on percentage breast weight were observed with any of the peptide treatments throughout the study.

With the gut weights (App. 19, Table 5), significant increases were observed on day 23 with Fraction 20 (Rockdale >100kDa) and Fraction 21 (Rockdale crude peptide) with protease compared to the control chicks. However significant increases in gut weights were also observed in control PBS and control PBS + protease treated chicks on this day negating any peptide fraction specific effects on gut weight.

The peptide treatments administered did not influence the length of any of the intestinal segments, (duodenum, jejunum, ileum and caeca) throughout the study.

Results from Chick evaluation study 4: Delivery of MBM-derived peptides to day old chicks following peptide treatment with a protease.

Throughout these studies peptide fractions isolated from Throsby MBM, did not impact on chick growth and performance, when compared with the control chicks, when administered both with and without protease treatment.

Peptide fractions isolated from Rockdale MBM, within the size range of 3-100kDa with and without protease treatment, and the >100kDa fraction with protease treatment, significantly increased percentage breast weights at day 23. No other chick parameters were affected.

From these studies the peptide fractions isolated from the current sample of Throsby MBM have no effect on chick growth and performance. However Rockdale derived MBM peptide fractions have demonstrated potential to enhance percentage breast weights in 3 week old chicks. The pre-treatment of the peptide fraction with protease did result in improved percentage breast weights with both the 3-100kDa and >100kDa preparations, however in the former this was not significantly different from the results seen when the 3-100kDa fraction was delivered in the absence of protease.

Discussion/Conclusion

Focus area 1: MBM extraction, fractionation and characterization

For this study of bioactive proteins and/or peptides in MBM a relatively gentle procedure was designed for the extraction and fractionation of water-soluble protein(s) or peptide(s) in an environment broadly compatible with protein and peptide bioactivities. Protein/peptide structure and bioactivity functions are highly susceptible to temperature and pressure effects. It was therefore notable that, despite the harsh rendering conditions of the high temperature and pressure that MBM is subjected to through rendering, significant protein and peptide was released from rendered and dried MBM using a water-based extraction procedure. This mild extraction approach may be the basis of a procedure that could be applied in scale-up for commercial application. Most importantly, the recovered protein in these extracts had demonstrated bioactivity. The results of the experiments revealed that proteins extracted from the low temperature rendered preparation (Rockdale) once fractionated and enriched by size-exclusion partitioning gave enhanced feed-growth in meat chicks (see next section - Focus Area 2).

Notably proteins/peptides from MBM revealed that the fraction of less than 100kDa extracted from the low temperature rendered preparation (i.e. Rockdale) promoted feed-growth enhancement, while protein and peptide extracts of less than 100 kDa prepared from a higher temperature rendering procedure (i.e., Throsby) were without feed-growth benefit. This might suggest that the differences in rendering temperature were responsible for the respective presence/absence of feed-growth bioactivity. Given the well-known temperature sensitivity for protein primary, secondary, tertiary and quaternary structures and their functional enzymatic, cofactor and protein-protein interactions, it is reasonable and tempting to propose that this is the explanation for the growth difference between formulations. However, before this can be established definitively a number of other possible differences between the preparations need also to be considered. Namely, the findings here are from only a single batch of each formulation, and undoubtedly there are batch to batch differences between preparations, and the starting raw materials. Furthermore, as raised earlier there are general differences in the animal ages used in the two formulations. In addition there were also differences in the extractions procedures performed here, for the molecular weights fractions for Rockdale MBM (i.e. 0-3, 3-30, 30-100 and 3-100 kDa) versus the Throsby MBM (i.e., 0-50, and 50-100 kDa) preparations. Whilst it is unlikely that the observed functional differences between the formulations for feed growth effects is due to their varied fractionations, this cannot be totally ruled out, particularly if the growth enhancing

activity is complex (i.e., relies on more than 1 molecular species, or perhaps a series of protein or protein fragment aggregates). There were various reasons why it was decided to do these exploratory fractionations in this manner. i) In order to cast a wide set of fractionation conditions to improve the chances of success in bioactivity detection, ii) with the formulations available, enable broad comparison of the two formulation groups with *in vitro* studies, and with *in-ovo* and feeding regimes, iii) enable their examination within the confines of the labour-intensive animal-base studies, and iv) provide a study of the Throsby MBM with some unique aspects that were suitable for the associated research student project. Further studies would provide the underlying reasons for the observed bioactivity in Rockdale MBM but absence from Throsby MBM, and clarify whether this difference is indeed due to the higher temperature in rendering by the Throsby procedure. Further analysis should include additional batches of the two formulations and identical fractionations i.e., with 3-100 kDa and 3-30, and 30-100kDa Rockdale and Throsby MBM extracts.

Considering the most prominent proteins in MBM preparations it was not surprising that by broad proteomic assessment of the extracts from the Rockdale and Throsby preparations collagen was the most commonly detected protein. Collagen is the most abundant protein of mammals and comprises approximately 25-35% of the total body protein content (Di LulloDagger *et al* 2002) and is even represented in the most resilient of tissues, skin, bone and teeth, where the predominant collagen in those tissues is collagen type 1, as identified in the MBM by mass spectrometry. Collagen comes in a variety of forms and is the most abundant protein in many tissues, and helps to maintain the integrity of the tissues via its structural support, and interactions in cells, in support of proteins and factors that promote cell viability and development. The intact collagen isoforms are relatively large and fibrous proteins, forming extremely large fibrils that resist protease disruption and under the temperature and pressure conditions of meat rendering would aggregate even further and most likely present in the MBM as aggregated broken fibrils and fragments. Other proteins identified in the MBM included calmodulin, AHNAK nucleoprotein, histone, and beta-casein. As with the collagens, these proteins are in abundance in animal tissues and are from either a cell support, extracellular matrix or nuclear (e.g., the DNA strand-associating histone protein) compartments. Additional sequences of significant protein fragments were also identified, and this is significant because it demonstrates that the rendering process does not completely denature all of the proteins in the product. Likely, these and other far less abundant proteins become entrapped and protected by the large aggregates of the abundant fibrous proteins, such as collagen. This provides a basis to explore MBM as a source of protein-based bioactivity, and is supported by the finding of positive growth bioactivity in the animal feed experiments.

The results of the identification study also demonstrated that there were differences in the proteins derived from the MBM provided by the two rendering plants. These differences may reflect the source of the material used, the nature of the rendering process, or simply batch-to-batch variation. Given that only one sample from each plant was available for analysis, it was not possible to comment further on the detail of this variation. However, it was notable that the high temperature rendering process used by Throsby correlated with a lower protein recovery. As noted elsewhere, the Throsby plant also generated MBM of a higher relative ash content, and this would also contribute to lower protein recovery.

Although the range of proteins identified were limited it should be emphasised that bioactivity can be present in a fragment or peptide that is derived from a parent protein, but does not

necessarily relate to the native function of that protein. Furthermore, we know from other studies that often highly active proteins, such as the cytokines, chemokines and growth factors, are present at less than a 1 parts per billion relative to the most abundant, but less bioactive proteins, such as collagen, albumin and fibrinogen. Hence we cannot rule out that the bioactivity observed in the experiments we conducted, may be present in trace amounts, and would therefore require greater levels of enrichment before identification was possible. Guided by the sensitive *in vivo* growth-response of the bioactive fractions tested in feed supplement studies, it is now feasible in follow-up studies to firstly enrich and purify this bioactivity further and thus determine to what maximum level this bioactivity can enhance growth response and identify the causative agent(s).

Focus area 2: Chick evaluation of MBM peptides.

Discussion of In-ovo studies

Initial *in-ovo* studies were designed to identify a positive standard that demonstrated increased chick growth compared to control birds, against which the performance of chicks receiving candidate MBM-derived peptides could be compared. Despite publications indicating that each of the test positive candidates (except IGF-Long R3 and whey protein) positively impacted on chick growth following *in-ovo* delivery, we only observed a positive impact with the delivery of glucose. As mentioned in the results section, the number of samples (n=5) taken on each observation day may have limited our ability to identify any effect. However it is also possible that many factors specific to the breeder flocks from which the embryos were sourced may have also influenced the effect of a candidate positive on the embryo/chick (discussed further below).

While several delivery methods for the actives were tested in initial studies (experiments 0109 and 0209), it became apparent that to use an *in-ovo* administration method that was already in use within the industry, was imperative. Hence from the third *in-ovo* study (Experiment 0309) delivery into the amnion at 18 days of embryonation, was the method employed.

In experiment 0309 the delivery of 50mg glucose into the amnion of 18 day old embryos increased mean body weight throughout the 3 week chick grow-out by 5% compared with the control chicks. These chicks also had higher mean breast weights (which was statistically significant overall when comparing the male chicks) than the control chicks. Further *in-ovo* administration of 50mg glucose significantly increased gut weights from 4 days until 21 days post hatch, by an average of 8%, compared with the control chicks. *In-ovo* delivery of 50mg glucose was also assessed in the subsequent experiment 0110, and it was again seen to increase chick mean body weights by 4% throughout the study (not statistically significant), breast weight were increased by 9% overall (not statistically significant) and percentage breast weight increased significantly (by 4.4%) at seven days of age. However in this study glucose administration did not influence gut weights. From these outcomes we recommended that the *in-ovo* delivery of 50mg glucose into the amnion of 18 day old embryos be used as a positive control.

Unfortunately in both experiments 0210 and 0310 where the *in-ovo* delivery of MBM-derived peptides was assessed, the *in-ovo* delivery of glucose did not generate the enhanced chick performance as seen in experiments 0309 and 0110. The reasons for this have not been

accurately identified however some possible influences may include the site of deposition of the substance with *in-ovo* administration, and characteristics of the parent breeder flock from which the embryos were sourced.

With *in-ovo* administration the site of deposition of the active into the egg may have varied between experiments, resulting in different outcomes in chick performance. As deposition is inside the egg, its exact site cannot be monitored without breaking out the embryo after delivery. However we have performed several test injections with coloured dye to assess the site of deposition on *in-ovo* administration, with repeated success for deposition into the amnion.

Other variables, including overall energy and protein reserves of the embryos/chicks which is largely influenced by the breeder parent flock, including their age and diet, may alter embryo/chick protein/energy status, impacting on the potential for glucose to enhance early chick growth. All embryos originated from the same supplier, with a request for the breeder parent flock to be aged between 30 to 35 weeks (the period of maximum laying/fertility and hatchability performance). However it was not possible to source the embryos from the same parent/breeder flock for each experiment. Similarly aspects of breeder flock management, for example diet, may have differed, which we are not aware of nor have any control over. As Uni and Ferket (2004) indicate, the chick experiences very high demands for energy during hatching and throughout the early post-hatch period. These demands will be met initially from the yolk, and then increasingly from the diet as the chick transitions onto the solid diet. If through the age of the parent flock and their diet the embryo and chick have a superior supply of energy, the administration of glucose during late embryogenesis may not promote chick growth to the level that would be seen in embryo/chicks experiencing a greater energy deficit at that time. As separate batches of embryos were used for each experiment differences in these parameters may have influenced the experimental outcomes.

Without further comparisons of some of these possible parameters and their potential influence on the effect of *in-ovo* delivered glucose on chick growth and development, we are unable to specifically identify the causative factors resulting in the lack of response seen in Experiments 0210 and 0310. However as the glucose administration is providing a source of energy rather than bioactivity its influence is unlikely to be through functions beyond fundamental nutrition (Meisel, 2007). Therefore the lack of response of the chicks to glucose in these studies may not negate the outcomes observed in chicks receiving the peptide fractions, if comparison is made to the control and vehicle control birds, as presented in the experimental results.

The *in-ovo* administration of peptide fractions extracted from MBM from Rockdale and Throsby rendering facilities did not enhance chick weights, breast weights or gut weights in experiments 0210 and 0310. However in several instances in Experiment 0210 the percentage breast weight was reduced in treated chicks. This was observed with each of the Rockdale peptide fractions on day 7 post-hatch.

Discussion of Post-Hatch studies

While not being statistically significant, administration of Rockdale peptides within the size range of 3-100kDa to chicks during their first week after hatch, did generate notable upward

trends for Cobb chick mean body weight (increases ranging from 6.8-9.6%) and increased mean breast weight (ranging from 9-13%) through to 4 weeks of age in experiment 0410.

The outcomes from experiment 0410 called for a closer assessment of the Rockdale 3-100kDa peptide fraction. Therefore in experiment 0610 the 3-100kDa treatment was administered, and two further treatments from within that fraction range of 3-30 and 30-100kDa, to Ross broiler chicks. A treatment of all peptides from the Rockdale MBM was also included. Despite the batch of birds in this experiment being affected by an Infectious stunting syndrome, chicks receiving Rockdale 3-30kDa and 3-100kDa fractions generated increases in the order of 7-9% mean body weight at 3 weeks of age. Mean breast weights, were also notably (but not statistically significant) increased with these peptide fractions. While these treatments did not generate any notable changes to gut weights, the length of the jejuna and ileal intestinal segments were significantly increased at 4 weeks of age compared to the control chickens.

The use of the Cobb and Ross line of bird in these studies incorporates the two major boiler lines used in the Australian broiler industry. The Ross line was also employed in experiment 0610 due to anecdotal evidence that its growth performance is more sensitive to protein than the Cobb line of bird. Taken together the outcomes from these experiments strongly support the potential for peptides within the 3-100kDa fraction of Rockdale derived MBM to possess bioactivity that can upregulate broiler chick performance. In the final experiment involving the Rockdale fractions (Experiment 0211) the 3-100kDa treatment significantly increased percentage breast weight at 3 weeks of age. No other significant effects were observed

In the two final experiments (experiment 0111 and 0211) peptide fractions were pre-treated with protease, to promote the release of other peptides, which may possess bioactivity, from parent proteins and peptides within the test fractions. Protease pre-treatment of the Throsby peptide fractions prior to their administration to the chick did not improve chick performance. Similarly with the Rockdale treatments, no benefits were seen in the protease treatment of the peptide fractions for mean bird weights, gut weight or intestinal length. However in this experiment the Rockdale 3-100kDa treatment significantly increased percentage breast weight compared to the controls at 3 weeks of age. Protease treatment of this same preparation also achieved this outcome, though it was not significantly different from administering the peptide fraction alone. Where protease treatment did have an effect was with the Rockdale >100kDa preparation, which also significantly increased 3 week percentage breast weight, compared to the control birds. In contrast to this when the Rockdale crude peptide preparation was pre-treated with protease, the percentage breast weight was significantly reduced compared with the controls. Hence thorough examination of the impact of protease treatment on the peptide fraction needs to be undertaken to fully appreciate how that may affect bird performance.

The inclusion of a protease in a broiler diet is designed to improved digestibility and chick growth. In experiments 0111 and 0211 this was not observed. However in these experiments the protease was only included in the peptide treatment, and a total volume of 1 ml of peptide + protease was administered throughout the study. Therefore this is a significantly lower administration level compared to its constant ingested when included in the diet, and the lack of a direct effect on bird performance in the protease control treatment was expected.

The MBM extracts for these experiments were each prepared separately. There were differences in their total protein content, which could only be determined retrospectively and hence the quantity of protein/peptide received by the chicks differed between experiments. As a result, the relative amounts of the apparent bioactive/growth enhancer also varied between experiments, which may have added to the variability seen in the growth responses observed between experiments. It is interesting to note that the peptide fractions Rockdale 3-100 and >100kDa administered in experiment 0610 had a higher protein concentration and 1.75 times higher respectively) than those used in experiment 0410 and 0211. In particular, with the Rockdale 3-100kDa fraction, the statistically significant results for increased jejuna and ileal lengths were observed in experiment 0610, when the greater quantity of bioactive was likely to have been administered. Similarly, when comparing the percentage increases in body and breast weights of the control and Rockdale 3-100kDa treated chicks across the experiment, the latter treatment tended to induce numerically higher mean weights in experiment 0610 compared to experiments 0410 and 0211, where the final amount of MBM-derived protein received by the chicks in experiment 0610 was at least twice the amount received in experiment 0410 and 0211. To date the peptide fractions have been delivered on a volume basis, with the protein concentrations being performed retrospectively. Therefore an assessment of the influence of protein dose of the Rockdale 3-100kDa on chicken performance is warranted.

In light of the overall outcomes of these experiments we propose that further work should be undertaken with Rockdale MBM to fully capitalise on the bioactive potential of its proteins/peptides. From the post-hatch studies the Rockdale 3-100kDa molecular weight range fraction has demonstrated the most frequent increase in chick growth and breast muscle deposition. This is in contrast to either of the peptide fractions sourced from Throsby MBM (>50kDa or <50kDa), which did not positively impact chick growth. To understand these differences and to further substantiate the bioactivity of the Rockdale derived 3-100kDa peptide preparation it will be necessary to determine whether there are inherent differences in the respective MBM materials or whether the different fractionation procedures are influencing the peptides extracted. Clarification of the bioactive protein and peptide types within the Rockdale 3-100kDa peptide fraction is also required so that these components can be targeted for isolation, concentration and delivery. From the outcomes of experiment 0610 some of the bioactives may be present in the 3-30kDa range of this larger fraction, however this requires further exploration. Further, once bioactive dose is optimized for best growth performance, it would be informative to determine if heat treatment of crude Rockdale MBM and/or the 3-100 kDa fractionated Rockdale extracts under conditions comparable to those experienced by the Throsby rendering procedure is accompanied by loss of bioactivity in the post-hatch chick feed response

As a side aspect, because we have demonstrated proof-of-principle water-soluble extraction of size partitioned bioactivity from Rockdale MBM, it is reasonable to ask whether the water soluble side product of the meat and rendering process, namely the stickwater of Rockdale processing, if fractionated similarly, also contains this bioactivity.

Proposed new work from outcomes of Project A.COP.0047

To identify the bioactive agents present in Rockdale MBM and their positive value for the poultry meat industry we propose new research work including studies designed to assess

1. The repeatability of the enhancement performance of chicks administered the Rockdale 3-100 and 3-30kDa preparations by comparing the peptides from the current preparations to those derived from new batch of Rockdale MBM. (using single sets of MBM extract fractions for multiple animal feed experiment assays, to reduce inter-assay variability).

2. A comparison of the growth response of chicks to graded dose rates of the further enriched Rockdale 3-100 and 3-30kDa fractions.

For application in the chicken meat industry both points 1 and 2 need to include a comparison of response with the Cobb and Ross broiler lines.

3. And compare the performance of chicks administered fractionated protein/peptides from the current batch of Rockdale MBM 3-100 and 3-30kDa preparation to the same size range fractions from Throsby MBM partitioning.

4. A comparison of protein and peptide types in Throsby and Rockdale MBM within the 3-100kDa weight range as an initial indicator of bioactive agents present in Rockdale MBM that may be absent from the Throsby MBM.

5. Biochemical identification and resolution of the proteins and/or peptide agent(s) in Rockdale 3-100kDa fraction responsible for the observed bioactivity.

6. Whether Rockdale prepared stickwater, partitioned and fractionated by 100kDa molecular weight exclusion contains the water-soluble growth-enhancing properties of extracts from MBM preparations.

Conclusion

In conclusion, the analysis of two sources of MBM-derived peptides in these studies has expanded our understanding of the characteristics of MBM, and its potential application as a source of bioactive materials. Collagen type-1 was the predominant protein in both samples of MBM. The rendering process influenced peptide availability, with more peptides being identified from the lower temperature rendering process compared to higher temperature rendering. Further the high temperature rendering process (Throsby) resulted in lower levels of energy and protein (including lower levels of each amino acid tested) and slightly higher levels of ash and lipid, compared to the low temperature facility at Rockdale. However the latter parameters may also be a reflection of the raw materials used for rendering at these plants.

To assess the bioactivity of these peptides following their administration to meat chickens, they were fractionated into pre-determined molecular weight size-ranges. Neither source of MBM-derived peptides enhanced chick performance following their *in-ovo* delivery 3 days prior to hatch. However, when delivered to the chick during its first week of life, the 3-100kDa fraction from the low temperature Rockdale facility initiated improved chick weights and breast weights in birds up to 4 weeks of age. Protease pre-treatment of the peptide fractions sourced from either rendering process to release additional bioactive agents did not demonstrate any additional ability to improve the growth responses in chicks.

In view of the demonstrated ability of the Rockdale 3-100kDa peptide fraction to increase broiler chick growth we recommend further characterisation and evaluation of those proteins and peptides. Identification of the bioactive agents will enable their targeted extraction and concentration to fully capitalise on their bioactive properties.

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